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New Tools for the site-specific attachment of proteins to surface

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Introduction

Protein microarrays in which proteins are immobilized to a solid surface are ideal reagents for high-throughput experiments that require very small amounts of analyte. Such protein microarrays ('protein chips') can be used very efficiently to analyze all kind of protein interactions en masse. Although a variety of methods are available for attaching proteins on solid surfaces. Most of them rely on non-specific adsorption methods or on the reaction of chemical groups within proteins (mainly, amino and carboxylic acid groups) with complementary reactive groups. In both cases the protein is attached to the surface in random orientations. The use of recombinant affinity tags addresses the orientation issue, however in most of the cases the interaction of the tags are reversible (e.g., glutathione S-transferase, maltose binding protein and poly-His) and, hence, are not stable over the course of subsequent assays or require large mediator proteins (e.g., biotin-avidin and antigen antibody). The key for the covalent attachment of a protein to a solid support with a total control over the orientation is to introduce two unique and mutually reactive groups on both the protein and the surface. The reaction between these two groups should be highly selective thus behaving like a molecular '*velcro*'.

Results and Discussion

The present work describes the use of protein splicing units (also called inteins) for the selective attachment of proteins to solid surfaces through its C-termini. In our a first approach we used "Expressed Protein Ligation" (EPL) [1,2] for the selective immobilization of proteins to a modified glass surface containing an N-terminal Cys poly(ethylene glycol) linker (Figure 1A). Key to this approach is the use of protein α-thioesters recombinantly generated using an engineered intein expression system. The protein α-thioesters are covalently attached by "Native Chemical Ligation" (NCL) to a glass surface modified with PEGylated thiol linkers **1** and **2** (Figure 1B). Two fluorescent proteins, EGFP (enhanced green fluorescent protein) and DsRed were used to test the suitability of EPL for selective protein immobilization. DsRed is a tetrameric red fluorescent protein and EGFP is a monomeric version of the green fluorescent protein. In both cases, the proteins are fluorescent only if their tertiary and quaternary structures are kept intact (DsRed shows red fluorescence only as tetramer). Thus, they were used as controls to test if the native architecture of these two proteins was altered during the attachment process. Both protein α-thioesters were readily expressed in *E. coli* using a modified Gyrase intein expression system. In order to facilitate the site-specific ligation of the fluorescent proteins onto a glass surface for the fabrication of protein microrrays, a glass slide was silanized with (3-acryloxypropyl)trimethoxysilane and reacted with a mixture of PEGylated thiols **1** and **2**, in a molar ratio 1:5, respectively. Linker **1** contained a protected N-terminal Cys for the selective attachment of the α-thioester protein through NCL and linker **2** was used as a diluent. Linker **1** also contains a longer PEG moiety than linker **2** (Fiure 1B) to ensure that the reactive Cys residue is available to react with the corresponding protein thioester in solution. After the glass derivatization was complete the protecting groups (N-Boc and S-Trt) of the Cys residue from linker **1** were removed by brief treatment with trifluoroacetic acid (TFA). The surface was rinsed, neutralized and quickly used for spotting (Figure 1C). As a control, a solution of EGFP with no α-thioester group was also spotted. The ligation reaction was kept for 36 h at room temperature, and the protein-modified slide was then extensively washed with phosphate buffer solution containing 0.2% Tween-20 (PBS). As shown in Figure 1, only specific attachment between the N-terminal Cys-containing surface and the protein α-thioester was observed. No fluorescence signal was detected where the control EGFP with no C-terminal α-thioester function was spotted. We also investigated the minimum protein concentration required for the affective immobilization of protein α-thioesters onto Cys-modified glass surfaces through NCL. Different concentrations of EGFP and DsRed α-thioesters were spotted onto a Cys-containing glass slide and incubated for 36 h. After extensive washing with PBS the slides were imaged for fluorescence (Figure 1C). As expected, the concentration of the protein was critical for efficient attachment of the corresponding protein α-thioester. In both cases, the minimun concentration required for acceptable levels of immobilization was found to be around 50 μM (≈ 1 mg/mL).

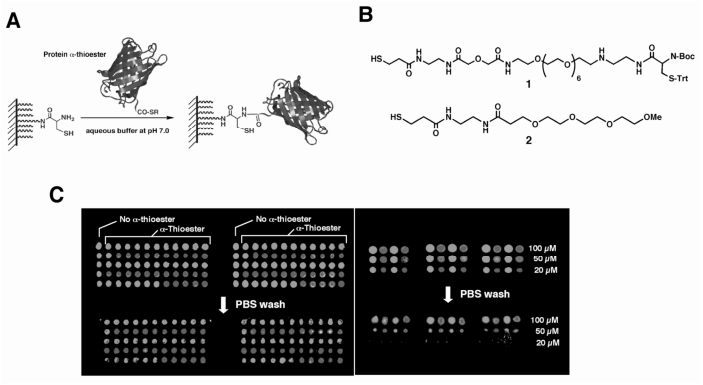
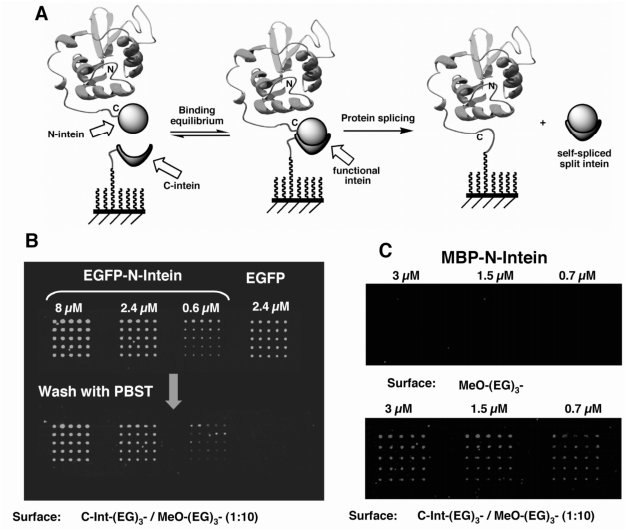


Fig. 1. A. Selective immobilization of proteins onto Cys-containing surfaces through Native Chemical Ligation. B. Chemical structures of linkers 1 and 2. C. Selective attachment of EGFP (lighter grey) and DsRed (darker grey) α-thioesteres onto a Cys-containing glass slide. Epifluorescence image of the glass slide after the protein spotting (top) and after PBS washes (botton). Spotting was carried out using 100 μM protein solutions (left panel) and with variable concentrations (from 100 μM to 20 μM) of protein α-thioesters (right panel).

More recently we have developed a new approach for the more efficient immobilization of proteins onto surfaces through their C-termini [3]. This new method is based on protein trans-splicing (Figure 2A) [4]. This naturally occurring process is similar to the protein splicing with the only difference that the intein self-processing domain is split in two fragments (called *N*-intein and *C*-intein, respectively). These two intein fragments alone are inactive. However, they can bind each other with high affinity under the appropriate conditions yielding a totally functional



splicing domain. In our approach, one of the fragments (*C*-intein) is covalently attached to the surface through a small peptide-linker while the other fragment (*N*-intein) is fused to the C-terminus of the protein to be attached to surface. When both intein fragments interact, they form the active intein which ligates the protein of interest to the surface at the same time the split intein is spliced out into solution. Key to our approach is the use of the naturally split DnaE intein from Synechocystis sp. PCC6803. In contrast with other inteins engineered to act as trans-splicing elements, which only work after a refolding step, the C- and N-intein fragments of the DnaE intein are able to self-assemble spontaneously ($K_d \approx 0.7$ μM) not requiring any refolding step.

Fig. 2. A. Site-specific immobilization of proteins through its C-termini to a solid surface by using protein trans-splicing . B. Immobilization and detection of EGFP-DnaE-N-Intein fusion protein. C. Immobilization and detection of MBP-DnaE-N-Intein fusion protein. Immobilized MBP was detected by incubating first with anti-MBP monoclonal murine antibody and then with anti-mouse IgG-TRITC conjugate.

We have successfully used this approach for the efficient immobilization of Maltose binding protein (MBP) and EGFP onto a C-Intein-modified glass slide (Figure 2B and 2C). In both cases the attachment was extremely selective with minimal background. Also, the minimun concentration required for effective protein immobilization was found

to be lower than 1 μM. This result demonstrates that this new method of protein immobilization can be easily interfaced with cell-free protein expression systems thus allowing rapid access to high throughput production of protein chips.

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